Mutation Research 811 (2016) 129–134

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Detection of *Pig-a* gene mutants in rat peripheral blood following a single urethane treatment: A comparison of the RBC *Pig-a* and PIGRET assays

Kazunori Narumi*, Yohei Fujiishi, Emiko Okada, Wakako Ohyama

Yakult Central Institute, Yakult Honsha Co., Ltd., 5-11 Izumi, Kunitachi-shi, Tokyo 186-8650, Japan

ARTICLE INFO

Article history:
Received 28 January 2016
Accepted 15 February 2016
Available online 2 March 2016

Keywords:
Pig-a gene
In vivo mutation assay
Peripheral blood
Reticulocyte
Urethane

ABSTRACT

The rat red blood cell (RBC) *Pig-a* assay has been recommended by an expert working group of the International Workshop on Genotoxicity Testing as a potential new method to evaluate *in vivo* gene mutations in regulatory genotoxicity risk assessments. In a collaborative study in Japan, an improved *Pig-a* assay using reticulocytes (PIGRET assay) with magnetic enrichment of CD71-positive cells was evaluated, and it was revealed that this assay could detect the mutagenicity of chemicals earlier than the RBC *Pig-a* assay could. To verify further the suitability of the PIGRET assay for an *in vivo* short-term genotoxicity screening test, a joint research study was conducted by the Japanese Environmental Mutagen Society, and 24 compounds were evaluated. One of the compounds evaluated in this study was urethane, a multi-organ rodent carcinogen. Urethane (250, 500, and 1000 mg/kg body weight) was orally administered once to 8-week-old male Crl:CD (SD) rats. Blood samples were collected at 1, 2, and 4 weeks after the administration and processed for the RBC *Pig-a* and PIGRET assays. In the PIGRET assay, the *Pig-a* mutant frequency (MF) significantly increased at both 2 and 4 weeks after the treatment of 1000 mg/kg of urethane. However, in the RBC *Pig-a* assay, a significant increase in the *Pig-a* MF was observed only at 1 week after the treatment with 500 mg/kg, but the MF value was within our historical control range; therefore, it was judged to be negative. These results suggest that the PIGRET assay might be useful for evaluating the *in vivo* mutagenicity more clearly than the RBC *Pig-a* assay after a single treatment of test compounds.

1. Introduction

The *Pig-a* assay is an *in vivo* gene mutation screening method using the endogenous X-linked phosphatidylinositol glycan class A gene (*Pig-a*) as a reporter of mutations [1–4]. *Pig-a* gene mutations are detected by the absence of antibody binding to erythrocyte surface glycosylphosphatidylinositol (GPI)-anchored proteins (e.g., CD59) based on a flow cytometric technique. This assay method is technically easier and more economical and practical for routine screening than the existing transgenic rodent (TGR) gene mutation assays [5].

In recent years, collaborative trial studies on the rat *Pig-a* assay have been conducted, and based on these and other results reported in the literature, an expert working group formed by the International Workshop on Genotoxicity Testing (IWGT) discussed and proposed how the assay should be conducted for the genotoxicity risk assessment of chemicals [6]. The working group recommended using a subchronic treatment protocol, ideally a 28-day daily dosing schedule similar to that recommended for the TGR gene mutation assay in OECD TG488 [7], which was required to ensure that sufficient frequencies of *Pig-a* mutant cells would accumulate in the peripheral blood cell population with repeated dosing. Short treatment schedules, however, are required to justify its use and recommended to conduct mutant analyses at approximately 4 weeks after the initiation of treatment. With regard to regulatory use, the ICH M7 guideline for the assessment and control of DNA-reactive (mutagenic) impurities in pharmaceuticals recommends the *Pig-a* assay to investigate the relevance of the bacterial mutagenicity assay results [8]. However, in most cases, it would be difficult to prepare a sufficient amount of impurities for a 28-day dosing schedule. Therefore, in such situations, a single treatment schedule is preferred.

Reticulocytes (RETs) turnover rapidly (within approximately one week) and express the mutant phenotype faster than the total population of red blood cells (RBCs) in peripheral blood [9]. In a Japanese collaborative study, an improved *Pig-a* assay in RETs

* Corresponding author. Fax: +81 42 577 3020.
E-mail address: kazunori-narumi@yakult.co.jp (K. Narumi).

http://dx.doi.org/10.1016/j.mrgentox.2016.02.004
1383-5718/

(PIGRET assay) with magnetic enrichment of CD71-positive cells was evaluated, and the findings revealed that it was possible to detect the *in vivo* mutagenicity of chemicals earlier than using the *Pig-a* assay in RBCs (RBC *Pig-a* assay) [10–15]. Based on this finding, the PIGRET assay is expected to be a useful and regular screening method to detect the mutagenicity of compounds in the early phase of drug development.

However, the number of compounds that have been tested using the PIGRET assay is limited; therefore, further research is needed to determine whether it has sufficient sensitivity to detect the mutagenicity of compounds after a single exposure. To this end, a joint research study was conducted by a task group belonging to the Mammalian Mutagenicity Study subgroup of the Japanese Environmental Mutagen Society (JEMS·MMS), and 24 compounds were comparatively evaluated by the RBC *Pig-a* and PIGRET assays. One of the compounds, urethane, a multi-organ rodent carcinogen [16–18], is generally considered to be genotoxic *in vivo*, but not *in vitro* [17,19–23]. Urethane is also known to require cytochrome P450-meditaed metabolic activation to exert its mutagenic effects and, therefore, is a key compound to evaluate the sensitivity of *in vivo* genotoxicity assays, especially for the detection of promutagens [17,18,24–26]. In the present study, we examined urethane in a time-course analysis of the *Pig-a* mutant frequency (MF) after a single treatment using both the RBC *Pig-a* and PIGRET assays.

2. Materials and methods

2.1. Chemicals

Urethane (ethyl carbamate, CAS No. 51-79-6) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and was dissolved in distilled water for injection (Fuso Pharmaceutical Factory, Inc., Tokushima, Japan) before use. *N*-ethyl-*N*-nitrosourea (ENU, CAS No. 759-73-9), which was used as a positive control, was purchased from Sigma–Aldrich Co., Ltd. (MO, USA) and dissolved in Dulbecco's PBS (Sigma–Aldrich Co., Ltd.). An anti-rat CD59 antibody (clone TH9, FITC-conjugated, 0.5 mg/mL), anti-rat CD71 antibody (clone OX-26, PE-conjugated, 0.2 mg/mL), and anti-rat erythroid marker (clone HIS49, APC-conjugated, 0.2 mg/mL) were purchased from Becton Dickinson and Company (NJ, USA).

2.2. Animals

Male Crl:CD (SD) rats, seven weeks old, were purchased from Charles River Japan, Inc. (Kanagawa, Japan), and acclimated for seven days. Male animals only were used in this study as discussed in the summary paper of this collaborative study [27]. The animals were housed two to three per cage in an air-conditioned room with a 12-h light/dark cycle and free access to food and drinking water. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the test facility prior to its implementation.

2.3. Dosage levels, treatment, and blood sampling

In a preliminary test of urethane (1000, 1500, 2000 mg/kg), a prone position and a decrease in locomotor activity were observed after a single treatment at all dose levels. Paralytic stupor was observed in the rats in the higher two dosing groups on the following day, and their body weights decreased more than 10%, probably because they were not eating. Based on these findings, the highest dose to be used for the urethane treatment in the main study was set at 1000 mg/kg, which was previously reported to induce micronucleated erythrocytes in rat bone marrow or peripheral blood following a single treatment (although the route of administration was i.p. in that study) [19,20]. We selected 500 and 250 mg/kg as the lower doses. Urethane was administered to six rats for each dose by a single oral gavage (10 mL/kg of body weight). The water and ENU (40 mg/kg), which were used for the vehicle and positive control groups, respectively, were administered in the same way as the urethane treatment, but three rats were used for ENU. Body weights were monitored throughout the experiment.

Blood samples of approximately 300 μL were collected from the jugular vein of all animals before dosing and at 2 days and 1, 2, and 4 weeks after the treatment and were immediately mixed with anticoagulant (EDTA–2 K). The samples obtained on day 2 were used for the micronucleus assay and the others were used for both the RBC *Pig-a* and PIGRET assays.

2.4. *RBC* Pig-a *assay, PIGRET assay, and %RET analysis*

The blood processing and analyses for both the RBC *Pig-a* and PIGRET assays, and the %RET analysis were performed according to the methods described in previous manuscripts [10].

For the RBC *Pig-a* assay, 3 μL of blood from each of the samples was suspended in 200 μL of PBS containing an anti-rat erythroid marker antibody (HIS49 antibody, 1:300 final dilution) and anti-rat CD59 antibody (1:100 dilution) and was incubated for 1 h at room temperature. Then, the samples were centrifuged at 1680 × *g* for 5 min, and the pellets were resuspended in 100–500 μL of PBS.

For the PIGRET assay, 100 μL of blood from each of the samples was suspended in 200 μL of PBS containing an anti-rat CD71 antibody (1:40 dilution) and was incubated for 15 min in a refrigerator. After being washed with 2 mL of 1 × IMa™ Buffer (Becton Dickinson and Company) and centrifugation (1680 × *g* for 5 min), the cells were mixed with 50 μL of BD IMag™ PE particle plus-DM (Becton Dickinson and Company) and incubated for 15 min in a refrigerator. The samples were enriched for CD71-positive cells by processing with a BD IMagnet™ magnetic stand (Becton Dickinson and Company) according to the manufacturer's instructions. The enriched samples were labeled with HIS49 and anti-CD59 antibodies in the same way as for the RBC *Pig-a* assay, with the exception that the incubation time was 30 min. The resulting pellets were resuspended in 500 μL of PBS.

For the %RET analysis, 3 μL of blood from each of the samples was suspended in 200 μL of PBS containing an HIS49 antibody (1:300 final dilution) and CD71 antibody (1:40 dilution) and was incubated for 30 min at room temperature. The samples were then centrifuged, and the pellets were resuspended in 100–500 μL of PBS.

A flow cytometric analysis was performed using a BD FACSVerse™ flow cytometer with BD FACSuite™ software (Becton Dickinson and Company). Approximately one million HIS-positive cells were acquired to determine the frequency of CD59-negative *Pig-a* mutant RBCs in the *Pig-a* assay. Approximately one million HIS-/CD71-double-positive cells were acquired to determine the frequency of CD59-negative *Pig-a* mutant RETs in the PIGRET assay. Twenty thousand HIS-positive cells were also acquired to determine the ratio of CD71-positive RBCs (%RET).

2.5. Peripheral blood micronucleus assay

One hundred microliters of blood from each of the samples was mixed with 200 μL of 10% neutral buffered formalin. Immediately before observation, the fixed cell suspension was mixed with an equal volume of an acridine orange (AO: 500 μg/mL) staining solution, dropped onto a glass slide, and covered with a coverslip. The slide preparation was observed using fluorescence microscopy (at 600× magnification with Blue excitation), and the number of RETs per 1000 erythrocytes and micronucleated RETs (MNRETs) per